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Determination of peroxidase activity in some fungi and in mycorrhizal and non-mycorrhizal Pinus sylvestris roots

ULLA AHONEN, ANNA MARI MARKKOLA, RAUNI OHTONEN, OILI TARVAINEN & HENRY VÄRE

Department of Botany, University of Oulu, Linnanmaa, 90570 Oulu

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Peroxidase activity was determined in certain mycorrhizal and decomposing fungi and in mycorrhizal and non-mycorrhizal Scots pine (*Pinus sylvestris* L.) roots using 3,3'-dimethoxybenzidine and p-methylaminophenol as substrates. 3,3'-Dimethoxybenzidine was found to be most useful, although there was a correlation between these substrates in all the material studied. p-Methylaminophenol more often gave negligible results. Only fresh sporophores can be used for peroxidase activity measurement, e.g. for taxonomic purposes, as the activity was usually much lower in dried specimens. Both intertaxon and intrataxon differences were observed. A peroxidase activity assay on fresh Scots pine root material can be used as an indicator of environmental stresses. Long-term cold storage reduces peroxidase activity significantly.

Keywords: peroxidase, mycorrhizal fungi, decomposing fungi, ectomycorrhiza, *Pinus sylvestris*

I. Introduction

Peroxidase activity is used as a non-specific stress indicator in plant tissues under various stress conditions. These include salt stress (Stevens et al. 1978), air pollution stress of various causes (Keller & Schwager 1972, Decleire et al. 1984, Huttunen 1988, Markkola et al. 1990) and hypoxia, freezing and pathogenic infection (Levitt 1972). Among other things, peroxidases catalyze the oxidation of phenols in the cell wall to compounds that are able to protect the cell, e.g. suberin and lignin (Fry 1986). Peroxidase activity has been investigated in some white-rot fungi (Lobarzewski 1986), where it takes part in lignin biotransformation in the wood substrate. Peroxidase activity may be involved in the ecological strategies of different groups of fungi, but no measurements have so far been reported on sporophores or aseptic cultures of mycorrhizal fungi. Peroxidases may also be of some taxonomic value in fungi, as substrate selectivity appears to distinguish a variety of algal groups, bryophytes, ferns and cycads (Siegel & Siegel 1986).

Chemical means of estimating potential stress in mycorrhizal roots would be desirable for improving the indicator value of the root parameters under different environmental conditions (Ohtonen et al. 1990), since these could prove more reliable than

parameters based on the morphology and anatomy of the mycorrhiza, as visual estimation of the condition of the mycorrhizal sheath is a subjective matter (Laiho et al. 1987) and there seems to be some controversy over the interpretation of results concerning the condition of mycorrhizae (Kottke & Oberwinkler 1986). Laborious light microscopical examination by measuring the mantle thickness and the formation of the Hartig net is often needed.

The aim of the present work was to evaluate the use of an assay of peroxidase activity in the case of mycorrhizal symbiosis. Pure culture mycelia and fresh and dried sporophores of some mycorrhizal and decomposing fungi were determined in order to evaluate the taxonomic diversity of fungi in relation to peroxidase activity. Mycorrhizal and non-mycorrhizal roots of Scots pine seedlings and fine roots of mature Scots pines collected from heavily polluted urban forest stands and less polluted forest stands were investigated. The effect of long-term storage on the activity in Scots pine roots was also evaluated.

II. Material and methods

1. Pretreatment of samples

Pure mycelial cultures of some ectomycorrhizal fungi (*Thelephora terrestris* (Ehrh.) Fr., *Suillus variegatus* (Fr.) O. Kunze, *Piloderma croceum* Erikss. & Hjortst., *Paxillus involutus* (Fr.) Fr., *Cenococcum geophilum* Fr., *Rhizopogon roseolus* (Corda) Th. M. Fries) were grown for two-three months on agar plates with MMN (modified Melin-Norkrans) nutrient solution (Marx 1969). Mycelia (30-70 mg fresh weight) were collected from the plate with tweezers and homogenized by grinding them in a mortar for 2 min in 2 ml potassium phosphate buffer (pH 6.3), and the homogenates were filtered and diluted with H_2O to a total volume of 3 ml.

Voucher specimens of sporophores of some mycorrhizal and decomposing fungi were obtained from the Botanical Museum of the University of Oulu. Sporophores were also collected from young stands of Scots pine (*Pinus sylvestris* L.) in Kittilä (67°37'N, 24°36'E) and from mature dryish stands of Scots pine around the city of Oulu (65°00'N, 25°30'E). About 200 mg of dried, pulverized pieces of caps was imbibed overnight in 2 ml potassium phosphate buffer (pH 6.3) in +6°C. This slurry and some caps of fresh sporophores (200-1500 mg fresh weight) thawed in 2 ml potassium phosphate buffer (pH 6.3) were homogenized with a rod homogenizer and filtered with distilled water to a total volume of 10 ml.

Aseptically germinated non-mycorrhizal and mycorrhizal Scots pine seedlings were grown on petri plates (Chilvers et al. 1986) in order to evaluate possible differences in peroxidase activity. The Scots pine seedlings were inoculated with *Piloderma croceum* and *Rhizopogon roseolus*. Ratio between the peroxidase activity measurements in the non-mycorrhizal (NM) and mycorrhizal (M) seedlings was calculated.

Other root samples for the peroxidase assays were collected in 10 mature dryish stands of Scots pine around the city of Oulu (65°00'N, 25°30'E) subjected to long-term deposition of urban air pollutants. Cores were taken from the entire depth of the mor humus layer with a stainless steel coring tool of diameter 3 cm in the autumn 1989 in 5 mature stands of Scots pine 30-40 km from the main emission sources and at 5 sites 1 - 5 km from the emission sources. The outermost sites will be referred to here as the non-polluted sites and the others as polluted sites, although the outermost stands are not actually free from deposition (Ohtonen et al. 1990).

The humus cores were stored in separate polyethylene bags in +6°C until used. The finest short and long roots (< 1mm in diameter, 30-110 mg fresh weight) of the Scots pines were collected separately from three humus cores per stand under a dissection microscope. The root tissues were homogenized by grinding them in a mortar for 2 min in 2 ml potassium phosphate buffer (pH 6.3), and the homogenates were filtered and diluted with H2O to a total volume of 3 ml.

2. Peroxidase assay

Homogenates prepared from mycelial cultures, dried and fresh sporophore material and pine roots were analysed for peroxidase activity (Siegel et al. 1989). Solutions for the peroxidase assay were prepared as follows: 3 ml H_2O, 1 ml buffer solution, 0.5 ml H_2O_2 (20 mM; for roots collected from mature pine stands 2 M) and 0.25 ml substrate. 10 mM 3,3'-dimethoxybenzidine (o-dianisidine) in the form of

dihydrochloride salt and 10 mM p-methylaminophenol were used as substrates. Other substrates (10 mM guaiacol and pyrogallol) were also tested, because the peroxidase enzyme is a complex enzyme group containing several isoenzymes. These substrates were rejected after preliminary tests because they showed little or no activity. All the reagents were kept at room temperature during the analysis. Being one of the most thermostable enzymes, peroxidase does not lose its activity to any significant extent even though not cooled during the procedure (Siegel et al. 1989). The peroxidase reaction was started by adding 0.25 ml of filtrate and the absorbance at 470 nm measured at 15 s intervals for 5 min using a Kinetics Soft-Pac module in a Beckman DU 64 Spectrophotometer.

The results were calculated in A min^{-1} g^{-1} fresh weight using the linear part of the reaction curve and the data analysed statistically using Student's t-test, one-way ANOVA and linear correlation analysis in the form of Microstat Statistical Programs.

III. Results and discussion

No activity was found in the mycelial cultures of any species analysed with any of the four substrates used. As the amounts of mycelia used in the analyses were relatively low, it would be worth testing larger amounts of mycelial biomass obtained using liquid cultures.

The fresh sporophores generally had higher peroxidase activity than the dried specimens (Table 1). This was tested with several species (n = 8) and with the genus *Russula* (n = 4). Peroxidase activity (3,3'-dimethoxybenzidine as substrate) was significantly higher in the fresh sporophores than in the dried ones ($n = 12$, $F = 8.89$, $p = 0.007$) and no correlation was found between them. This indicates that dried sporophores cannot be used for peroxidase analysis e.g. for taxonomical purposes. There was no difference between the fresh and the dried specimens using p-methylaminophenol as a substrate, but the activities were low, seldom departing from zero. 3,3'-Dimethoxybenzidine was a more effective subtrate, and no correlation was found between 3,3'-dimethoxybenzidine and p-methylaminophenol in the fresh sporophore samples. Thus it seems to be worth of using both substrates on fresh sporophore material.

No difference in peroxidase activity was found between the mycorrhizal and decomposing fungi in either the dried or the fresh material (Table 1). Some differences in peroxidase activity could be found between the taxons at the species-family level in the fresh material (Table 1). Peroxidase activity was especially high in fresh Russulaceae (*Russula paludosa*, *R. vinosa*, *R. xerampelina*) and Clavariadelphaceae (*Clavariadelphus ligula*), while Boleteceae and Tricholomataceae showed no activity. At the genus level, for example, *Lactarius helvus* showed no activity but *L. rufus* high activity when 3,3'-dimethoxybenzidine was used as a substrate (38.23 A min^{-1} g^{-1} Fw). Among the decomposing fungi there was no activity in Coprinaceae and Lycoperdaceae in fresh material.

The peroxidase activity in the non-mycorrhizal roots of the aseptically grown Scots pines was on average 2.77 (S.D. 1.8) and 1.64 (S.D. 1.66) times higher than that in the mycorrhizal roots (with 3,3'-dimethoxybenzidine and p-methylaminophenol as substrates, respectively). A high correlation was found between these two ratios (Fig. 1). The results suggest that non-mycorrhizal seedlings may suffer from nutritional or perhaps water stress.

The present peroxidase activity measurements are in accordance with the results of Mitchell et al.

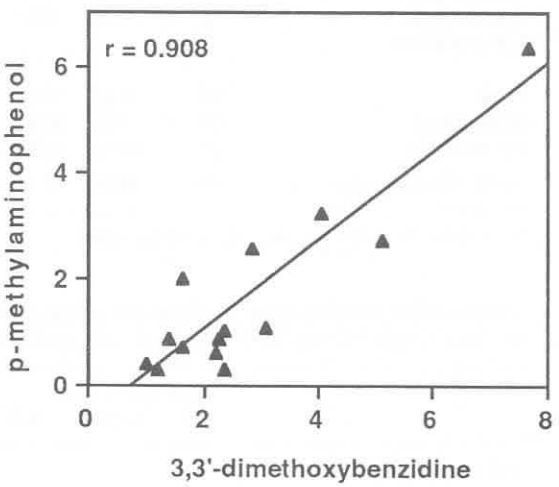

Fig. 1. Correlation between peroxidase activities determined from the roots of aseptically grown mycorrhizal and non-mycorrhizal seedligs using 3,3'-dimethoxybenzidine and p-methylaminophenol as substrates. The values were obtained by dividing the activities in non-mycorrhizal and mycorrhizal seedlings to give a ratio between non-mycorrhizal and mycorrhizal seedlings (NM/M ratio). n = 14.

Table 1. Peroxidase activity (A min^{-1} g^{-1}) in dried and fresh mycorrhizal samples and the decomposing taxons. The substrates used were 3,3'-dimethoxybenzidine (benz.) and p-methylaminophenol (phen.).

	Dried					Fresh				
		benz.		phen.			benz.		phen.	
	n	Mean	S.D.	Mean	S.D.	n	Mean	S.D.	Mean	S.D.
Mycorrhizal fungi										
Elaphomycetaceae	2	0.12	0.17	0						
Clavariadelphaceae	2	0.95	0.66	2.08	0.66	1	104.24		20.44	
Cantharellaceae	2	0	0	0	0					
Thelephoraceae	5	0.09	0.12	0.18	0.25					
Boletaceae	5	0	0	0	0	6	0	0	0	0
Paxillaceae	3	3.06	2.28	2.68	2.17					
Gomphidiaceae	2	0	0	0	0					
Hygrophoraceae	1	0.45		0						
Tricholomataceae	6	0.06	0.08	0.13	0.27	2	0	0	0	0
Amanitaceae	2	0	0	0	0	2	0.20	0.28	0	0
Cortinariaceae	18	4.97	19.84	0.79	1.83					
excl. *C. traganus*	15	0.30	0.50	0.11	0.41	12	1.84	2.92	0	0
C. traganus	3	28.47	48.49	3.68	3.34					
Russulaceae	13	0.85	0.98	0	0	9	47.58	44.71	0.83	0.87
Lactarius	9	1.32	1.68	0	0	4	19.29	23.93	0	0
Russula	4	0.11	0.24	0	0	5	70.21	46.13	1.02	1.00
Rhizopogonaceae	2	0	0	0	0					
Sclerodermataceae	4	0	0	0	0					
Decomposing fungi										
Helvellaceae	1	0		0						
Tricholomataceae	10	33.52	81.92	1.93	4.08					
excl. *Cantharellula*	8	0	0	0	0	5	0.59	0.82	0	0
Cantharellula	2	167.61	124.31	9.66	0.98					
Amanitaceae	2	0	0	0	0					
Strophariaceae	2	0	0	0	0					
Coprinaceae	2	4.75	6.72	0	0	2	0	0	0	0
Lycoperdaceae						1	0	0	0	0
Total	84	6.13	32.18	0.60	1.89	40	13.95	31.86	0.70	3.24
Mycorrhizal	67	1.78	10.81	0.41	1.21	32	17.34	34.89	0.87	3.62
Decomposing	17	24.59	69.70	1.39	3.48					
excl. *Cantharellula*	15	0.79	2.74	0	0	8	0.37	0.69	0	0

n = number of samples, S.D. = standard deviation

Table 2. Effect of cold storage (+6°C) on peroxidase activity (A min^{-1} fwg^{-1}) in Scots pine roots. The significance (p) of the difference between fresh roots (September) and roots stored 4 months (January) is indicated (Student's t-test).

	September 1989			January 1990				
	n	Mean	S.D.	n	Mean	S.D.	t	p
Polluted stands								
3,3'-dimethoxybenzidine	2	34.44	19.40	2	6.10	1.33	2.06	0.088
p-methylaminophenol	2	3.04	3.44	2	0.76	0.04	0.93	0.226
Non-polluted stands								
3,3'-dimethoxybenzidine	3	8.93	4.50	3	5.17	2.74	1.24	0.142
p-methylaminophenol	3	2.73	0.90	3	0.50	0.02	4.27	0.006

n = number of samples, S.D. = standard deviation

(1986), who showed that this activity was higher in non-mycorrhizal than in mycorrhizal *Pinus* roots, whereas Ronald & Söderhäll (1985) did not find any difference between the two. Moreover, an increase in cell wall peroxidase activity in endomycorrhizal *Allium porrum* roots compared with non-mycorrhizal controls has been reported at the initial stages of fungal penetration in the root cells (Spanu & Bonfante-Fasolo 1988). The fungal symbiont in ectomycorrhizal associations does not penetrate host cells, however, suggesting that wall enzymes are not involved in mycorrhiza formation (Nylund 1988).

Peroxidase activity was significantly higher in the root samples collected from urban polluted stands than in non-polluted stands (40.79 and 7.23 A min^{-1} g^{-1} fresh weight, respectively, $n = 15$, $F = 57.74$, $p < 0.001$), when 3,3'-dimethoxybenzidine was used as a substrate. When p-methylaminophenol was used, the activities were distinctly lower on average. Peroxidase activity was again higher in the samples from the polluted area, but the difference between the sites was not significant. The activities obtained with the two substrates for the same sample correlated significantly ($r = 0.643$, $p < 0.001$).

Peroxidase activity in the Scots pine roots decreased considerably during the cold storage period of 4 months (Table 2), the average value of two determinations on the samples collected from the polluted area decreasing to about one-fifth with both substrates. The samples collected from the non-polluted stands showed only a slight decrease when 3,3'-dimethoxybenzidine was used as a substrate, but the decrease in activity was significant when p-methylaminophenol was used as a substrate ($p < 0.01$). Long-term storage of humus cores seems to destroy or inactivate peroxidases in the roots. Thus peroxidase activity determination seems to be unusable in the case of such samples. This statement is based on only a small number of measurements, however, and further investigations into the effect of deep-freezing are needed.

The results show that peroxidase activity measurements using 3,3'-dimethoxybenzidine as a substrate could be used with fresh material 1) for taxonomical research on fungi and 2) as an indicator of pollution and other stress factors affecting Scots pine roots. As some mycorrhizal fungus species show high activity, it is possible that this could also be detected in the ectomycorrhizae formed by these species.

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